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Waltzing transporters and 'the dance macabre' between humans and bacteria

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Abstract | Multidrug-resistance efflux pumps — in particular those belonging to the resistance-nodulation-cell-division (RND) family of transporters, with their unusually high degree of substrate promiscuity — significantly restrict the effectiveness of antibacterial therapy. Recent years have heralded remarkable insights into the structure and mechanisms of these fascinating molecular machines. Here, we review recent advances in the field and describe various approaches used in combating efflux-mediated resistance.

Nosocomial
Acquired or occurring in a hospital.

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The word *cell* comes from the Latin *cella*, meaning a small room. As with tidily kept rooms, cells of all living organisms, be they eukaryotes, prokaryotes or archaea, use special types of membrane transport proteins, often referred to as 'molecular vacuum cleaners', to keep their interiors free from toxic by-products of cellular metabolism as well as a variety of environmental toxins. The 'vacuum cleaner' analogy goes deeper: these proteins, called multidrug-resistance (MDR) efflux pumps, are remarkably indiscriminate toward the molecular 'dust' that they can capture and expel from a cell. A single MDR transporter might handle literally hundreds of structurally diverse compounds. This substrate promiscuity makes MDR transporters a fascinating subject of research, but also renders some of them a serious impediment to human healthcare, as many of their substrates are clinically useful drugs. The very first MDR transporter to be discovered, human P-glycoprotein (P-gp)[1–3], renders tumour cells resistant to anticancer therapy. MDR pumps confer clinical resistance to antifungal[4] and antiprotozoal agents[5]. In bacteria they diminish the effectiveness of all classes of antibiotics[6,7].

MDRs come in many shapes and forms. They can be found among transporters from five evolutionarily distinct families[8–13], differing significantly in molecular architecture and transport mechanisms. Efflux can be driven by ATP hydrolysis, as in the case of transporters from the ATP-binding cassette (ABC) class, or coupled with transport of ions, most frequently protons (transporters of all other classes). They can function as monomers or oligomers and work alone or as a part of multi-protein complexes. For some, their main natural function is protective, whereas others extrude drugs only when presented with a choice 'to efflux or to die'[14,15]. The most dedicated of 'cellular guards' can be identified relatively easily by their broad substrate spectrum, and their high basal level in cells, which can be further increased in the presence of drugs. In addition, they usually work in concert with other components of nonspecific cell defence. There are several instances where this organized defence provides an almost impenetrable barrier for attack with therapeutic agents. Gram-negative bacteria prove to be one such case[16,17].

Unmet clinical need and the curse of efflux

In the clinic, most classes of currently available antibiotics (for example, macrolides/ketolides, oxazolidinones, lipopeptides, glycopeptides, rifamycins and streptogramines) are ineffective against Gram-negative bacteria and are used to treat Gram-positive bacterial infections only. This is because Gram-negative pathogens have a high level of intrinsic antibiotic resistance. Some bacteria are particularly notorious in this respect. Among them is *Pseudomonas aeruginosa*, a common nosocomial pathogen, the causative agent of many life-threatening infections and the major reason for the shortened lifespan of people with cystic fibrosis. *Pseudomonas* infections[18] can be successfully treated by only a few of the 160 approved antibiotics, such as the members of the fluoroquinolone, β-lactam or aminoglycoside classes.

That intrinsic efflux is the cause of non-susceptibility to antibiotics is evident from the fact that deletion of major constitutively expressed MDR efflux pumps renders *P. aeruginosa* and many other Gram-negative bacteria as sensitive to antibiotics as the much more susceptible Gram-positive pathogens[6,19]. As might be

expected and is well-documented[7], enhanced efflux due to overexpression of MDR pumps has a prominent role in acquired resistance as well. It is important to note that although MDR efflux is not the only mechanism of resistance to a particular agent, numerous *in vitro* studies provide strong evidence that in the absence of efflux the emergence of unrelated resistance mechanisms might be significantly reduced[20–22].

This phenomenon of 'Gram-negative non-susceptibility' is seen not only for approved antibiotics, but also for drugs in clinical development and for compounds at various stages of preclinical research[23]. Its pervasiveness is particularly unfortunate given that most of these compounds have the potential to be truly broad-spectrum, as they inhibit the activity of targets that are well-conserved in both Gram-positive and Gram-negative bacteria.

In drug discovery, it is commonly observed that the primary hit-rate in random antibacterial screens using large libraries of synthetic compounds or natural products is up to 1,000-fold lower against Gram-negative than for Gram-positive bacteria. The hit rate is significantly increased when wild-type strains are substituted with mutants lacking efflux pumps[24]. So, it is clear that MDRs can successfully handle thousands of substrates. The result of this intrinsic efflux is an inadequate number of primary hits, which are the starting point for the discovery of new agents. For a while, using efflux mutants for screening to identify more hit compounds was a strategy extensively used in various companies. However, no new agents emerged and the pipeline of 'Gram-negative' antibiotics dried up.

Given this background, the emergence of resistance to the few antibiotics that are still useful for the treatment of Gram-negative infections is particularly disturbing and suggests that strategies should be implemented to preserve the clinical effectiveness of these agents. One possibility is to interfere with efflux pumps through the use of small-molecule inhibitors. Such inhibitors, when used in combination with antibiotics, will increase antibacterial potency, expand the spectrum of antibacterial activity, reverse resistance and dramatically reduce the rates of resistance development[23,25].

An alternative approach is to identify and develop compounds that avoid efflux pumps altogether[26]. This might be a more elegant and appealing strategy from the development perspective (it is much easier and cheaper to develop one drug than a combination product), but seems to be less feasible. The successful candidate would need to evade the numerous recognition sites of MDR transporters, be able to penetrate the two very different membranes of Gram-negative bacteria[27], and still maintain its antibacterial activity. It is interesting that the only antibiotics that are not subject to efflux in Gram-negative bacteria are some β-lactams[28]. To kill, these antibiotics need to cross only one of the two membranes, because their targets, the penicillin-binding proteins, are located in the periplasm. Therefore periplasmic targeting might be preferential to cytoplasmic targeting, because identification of a compound that reaches and hits a cytoplasmic target without being expelled by any of the MDR transporters in Gram-negative bacteria is highly unlikely.

RND permease superfamily
The resistance-nodulation-cell-division (RND) family of transporters are found ubiquitously in bacteria, archaea and eukaryotes. In accordance with the Transporter Classification (TC) system (www.tcdb.org]) approved by IUBMB (International Union of Biochemistry and Molecular BiologyY) its TC number is 2.A.6.

Efflux mechanisms

Efflux is most effective when working in cooperation with other resistance mechanisms. Reduced uptake across the outer membrane of Gram-negative bacteria, which is a significant permeability barrier for both hydrophilic and hydrophobic compounds, constitutes such a mechanism[29,30]. To take advantage of the reduced uptake, some Gram-negative MDR pumps extrude their substrates across the whole cellular envelope and out of the cell. For the remainder of this review, we refer to this process as trans-envelope transport[31].

The MDRs that function in this way belong to the resistance-nodulation-cell-division (RND) family of transporters[12]. These pumps possess an astonishing breadth of substrate specificity, and in this respect surpass even the notorious ABC-transporters, which are major hurdles for the effectiveness of anticancer therapy[32]. RND pumps can recognize and extrude positively or negatively charged and neutral molecules, substances as hydrophobic as organic solvents and lipids, and compounds as hydrophilic as aminoglycoside antibiotics. They are a ubiquitous family, whose members are distributed across various phylogenetic kingdoms. Several representatives of the RND permease superfamily are encoded in the human genome, though their similarity to bacterial RNDs is negligible (16% identity). Examples of human RNDs include the Niemann–Pick C1 (NPC1) protein, localized in lysosomal membranes and apparently involved in intracellular cholesterol transport[33], and the homologue of *Drosophila* morphogen receptor Patched, thought to be crucial in the suppression of basal cell carcinoma[12,34]. Given the low similarity between bacterial and human RNDs, the identification of highly selective inhibitors of bacterial pumps devoid of mechanism-based mammalian toxicity seems to be feasible.

The past few years have seen the publication of an extraordinary amount of structural information on bacterial RND transporters. Several high-resolution structures of the AcrB efflux pump from *Escherichia coli*, with and without co-crystallized substrates, as well as several mutant AcrBs, have recently been published[35–40]. As discussed below, RND transporters from Gram-negative bacteria function as a complex with two other types of proteins, and X-ray structures of these have also become available[41–45]. Only the structure of the tri-partite complex (see later and FIG. 1) is lacking. This detailed structural information should dramatically facilitate the discovery of inhibitors of RND transporters to improve efficacy of antibiotics against problematic bacteria that are the cause of many life-threatening infections.

In this review we will summarize recent advances on the structure and function of RND transporters. It is important to emphasize that much research has also been devoted to understanding the complex regulation of efflux gene expression, which is usually governed by local and global regulatory mechanisms. To learn more about this topic, the reader is referred to several excellent reviews[46–49].

In addition, more information is becoming available regarding the significance of RND transporters in bacterial pathogenesis. Many transporters have been demonstrated to be essential for cellular invasion and

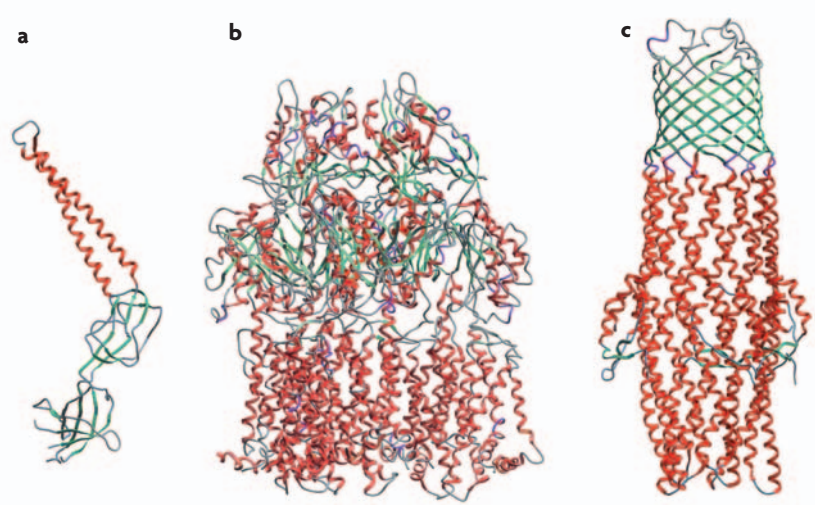

Figure 1 | **Structure of AcrA, AcrB and TolC proteins from *Escherichia coli*.** α-Helices and β-sheets are coloured in red and green, respectively. The figure shows a monomer of AcrA (**a**) and trimers of AcrB (**b**) and TolC (**c**). All proteins are shown to scale.

resistance to natural host substances, such as bile salts and specialized host defence molecules[50]. Some RND transporters from *P. aeruginosa* (and other relevant pathogens) are involved in controlling the balance of quorum signal molecules that are important for cell-to-cell communication, and for the establishment of persisting infections[51,52]. Interestingly, the Gram-positive organism *Mycobacterium tuberculosis* contains four RND transporters involved in controlling virulence[53]. A comprehensive review regarding this emerging 'physiological' role of RND transporters has recently been published[50].

True teamwork of RND-mediated efflux

To perform trans-envelope efflux, the inner membrane RND transporter works together with accessory proteins: the periplasmic protein, belonging to the membrane fusion protein (MFP) family; and the outer membrane channel, a member of the outer membrane factor (OMF) family. RND pumps and accessory proteins form large multi-protein assemblies that traverse both the inner and outer membranes of Gram-negative bacteria[31,54,55]. All components are essential for transport. Working together as a well-coordinated team, they achieve the direct extrusion of substrates across the entire cell envelope and into the extracellular medium.

The integrity of the outer membrane is crucial. When it is compromised, no resistance is seen, even in the presence of fully functional RND-containing complexes[16]. One might infer that these MDR transporters are, in fact, rather sluggish machines, relying heavily on restricted diffusion of effluxed compounds back into the bacterial cell, but no reliable kinetic measurements exist for RND transporters to support this point. An alternative explanation is that the outer membrane is simply the only barrier across which substrates are transported, and that RND transporters, unlike all other known drug pumps, are capable of capturing their substrates in the periplasmic space rather than in the membrane, or from the cytoplasm. X-ray crystallography illuminates these points.

Quorum signal molecules
Molecules that are released by the microorganisms as a means of monitoring population density. When these signal molecules reach a threshold concentration, the population density has attained a critical level or quorum, and quorum-dependent genes are expressed.

Trans-envelope pump architecture

High-resolution structures of all components of the efflux complex are now available. A steady stream of structural information, starting with the OMF TolC[45] protein from *E. coli* in 2000 and continuing to the present day, has produced a series of remarkable discoveries[38–40].

The best-studied AcrB transporter from *E. coli* serves as a prototype for all members of the RND family. AcrB is a protein of approximately 1,100 amino-acid residues that contains a trans-membrane domain (TMD), comprises 12 TM segments, and an unusually large periplasmic domain. Importantly, structural data have established that AcrB functions as a trimer (FIG. 1). Inside the membrane, monomers of AcrB, which we will refer to as protomers, have very limited contact with each other[35]. In the periplasm, by contrast, they assemble into an intricate 'mushroom'-like structure that protrudes about 70 Å from the inner membrane. The periplasmic portion of the AcrB trimer can be further sub-divided into the porter and the TolC docking domains. In the porter domain, neighbouring protomers form three large vestibules that are exposed to the periplasm. These vestibules lead to a spacious central cavity.

The structural features of the accessory proteins are consistent with their role in extending drug efflux across the outer membrane. The three-dimensional structures of three OMFs — TolC, OprM and VceC, from *E. coli*, *P. aeruginosa* and *Vibrio cholerae*, respectively — have been solved recently[44,45,56]. Despite very little sequence similarity, they are structurally conserved. Like AcrB, they form stable trimers organized into two barrel structures (FIG. 1). A 12-stranded β-barrel, 40 Å long, inserts into the outer membrane to form an open pore 30 Å in diameter. An unusual α-helical barrel 100 Å in length protrudes deep into the periplasm, where it reaches the TolC docking domain of AcrB. The lower half of this barrel is bound by an equatorial domain of mixed α/β-structure. The tip of the periplasmic end of the channel is closed in an iris-like manner by interacting loops of α-helices.

Biochemical and genetic data demonstrate that MFPs interact with both the RND pump and the OMF channel[57,58]. It is therefore proposed that the MFP stabilizes weak RND–OMF interactions, and promotes and maintains the tri-partite complex. Recently determined structures of MexA and AcrA, from *P. aeruginosa* and *E. coli* respectively[41–43], are consistent with such a function (FIG. 1). These MFPs seem to have a modular structure, with a long β-barrel domain connected to a lipoyl domain that is in turn attached to a long periplasmic α-helical hairpin. By forcing *E. coli* TolC to make a functional complex with the MexAB-translocase from *P. aeruginosa* (which otherwise is non-functional), it was established that key interactions between MFP and OMF are located in the equatorial domain and in the vicinity of the coiled coils of the TolC entrance[59]. In addition, MFP seems to possess significant conformational flexibility[60], which might be important to ensure the most advantageous interaction with TolC.

In modelling an 'open state' of the TolC entrance, there seems to be a perfect fit with the funnel-like opening of the TolC docking domain of AcrB[61]. The possibility

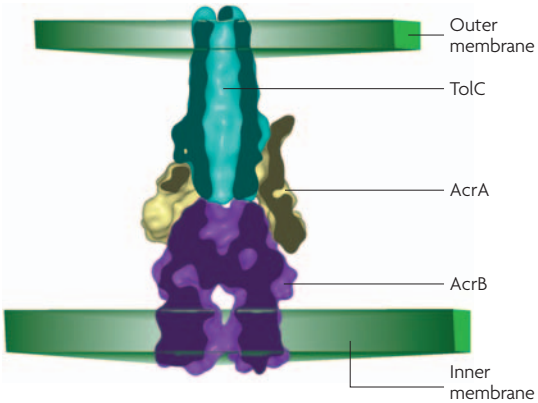

Figure 2 | **Fitted model of the *Escherichia coli* AcrAB–TolC tri-partite efflux complex.** The simplified shape of the protein surface was generated in ICM, a package of programs for molecular modelling and docking. Most of the AcrA trimers are removed by cross-section. There is significant evidence that the 'crater' in the fully assembled pump is connected to TolC, either directly or via an adaptor composed of AcrA subunits. The tubular TolC structure serves as an 'exhaust pipe' that penetrates the outer membrane, providing the exit route for the substrate efflux into the external medium. The opening indicated by the white colour in the AcrB-trimer leads to the periplasmic space.

exists that the MFP has an active role in opening of the TolC channel during drug transport. It is also possible that the 'open' state is the result of the AcrB–TolC interaction, and that the role of MFP is to keep both proteins in this fixed state.

The oligomeric state of MFPs still remains controversial. Soluble forms of AcrA and MexA have been found to be monomeric *in vitro*, but crosslinking of AcrA *in vivo* suggests that the MFP works as a trimer with its two other partners[62,63].

Although, many details remain to be clarified, the emerging architecture of the trimeric complex provides a structural basis for understanding trans-envelope efflux (FIG. 2). A substrate enters the tri-partite transporter through the appropriate inter-envelope 'substrate gate' and exits into the extracellular space through the 'exhaust pipe' of TolC.

RND pumps capture substrates in the periplasm

The possibility of periplasmic capture first arose in light of genetic and biochemical experiments[64–67], and was subsequently reinforced with the advent of the high-resolution structures of protein–ligand co-crystals.

Interestingly, co-crystals revealed several possible drug-interacting sites. In the first report several unrelated drugs were detected in the central cavity on the membrane–periplasm interface, prompting a model in which drugs first intercalate into the phospholipid bilayer and then diffuse laterally into the central cavity of AcrB[37,68]. Drugs observed in this site are in the vicinity of a few aromatic residues that seem to interact with them through hydrophobic and stacking interactions. However, the observed ligand–protein interactions seemed weak and are insufficient to explain substrate specificity. In fact, the few amino-acid-residue side-chains observed to interact with the ligands are highly conserved across various efflux pump proteins of broadly varying substrate recognition. Mutational analyses of AcrB and the related EmhB from *Pseudomonas fluorescens* nevertheless show that amino-acid residues in the central cavity do affect transporter-mediated antibiotic resistance[36,69].

In the next published structure of the same protein, an additional drug-binding pocket was detected in a prominent cleft on the surface of the periplasmic domain. This site might be fully exposed to the periplasm, with easy access for drug binding. The problem with this site is that it is not clear where the drug can go to once it is there. Mutations altering several amino acids within this site were also reported to affect RND-related antibiotic resistance[36,65,70]. One intriguing possibility is that this site might have a role in regulating the activity of the transporter by its substrates, rather than mediating the actual transport process directly.

Finally, a third structure revealed yet another, non-overlapping, multidrug-binding pocket, located deep inside the periplasmic domain[39]. This voluminous pocket is extremely rich in aromatic amino-acid residues that are capable of hydrophobic and stacking interactions. There are also a few polar residues that can form hydrogen bonds. Interestingly, the co-crystallized substrates doxorubicin and minocycline were found to interact with different sets of amino-acid residues. This finding fits very well with the rapidly emerging paradigm of a versatile multi-specific recognition pocket, which was originally proposed on the basis of results obtained with soluble multidrug-binding regulatory proteins such as BmrR[71,72], QacR[73,74] and PXR[75]. These studies demonstrated that different substrates can use different residues to bind in the same pocket; that the same substrates can assume multiple positions in the pocket; that two substrates can be bound simultaneously; and that this binding can give rise to negative or positive cooperativity. Numerous studies on P-gp, though not at the structural level, illustrate such versatility through the concept of the 'induced best fit', by which a substrate can provoke rearrangements in the pocket during binding[76,77].

The first high-resolution structure of the MDR ABC-transporter, Sav1866 from *Staphylococcus aureus*, has also been determined[78]. Though the crystals lack substrate, the location of the binding pocket can be easily identified on the basis of a variety of biochemical, mutagenesis and crosslinking experimental data[79,80], collected over many years of research on ABC transporters. The large chamber within the membrane that is formed by two TMDs opening to the extracellular milieu might also be accessible from the lipid phase at the interfaces between the two TMDs[81], constituting a drug-binding pocket for the MDR ABC transporters. The TM domains of P-gp do not contain any charged residues. Therefore, ligand–protein interactions are based solely on hydrogen bonding, hydrophobic and stacking interactions. The

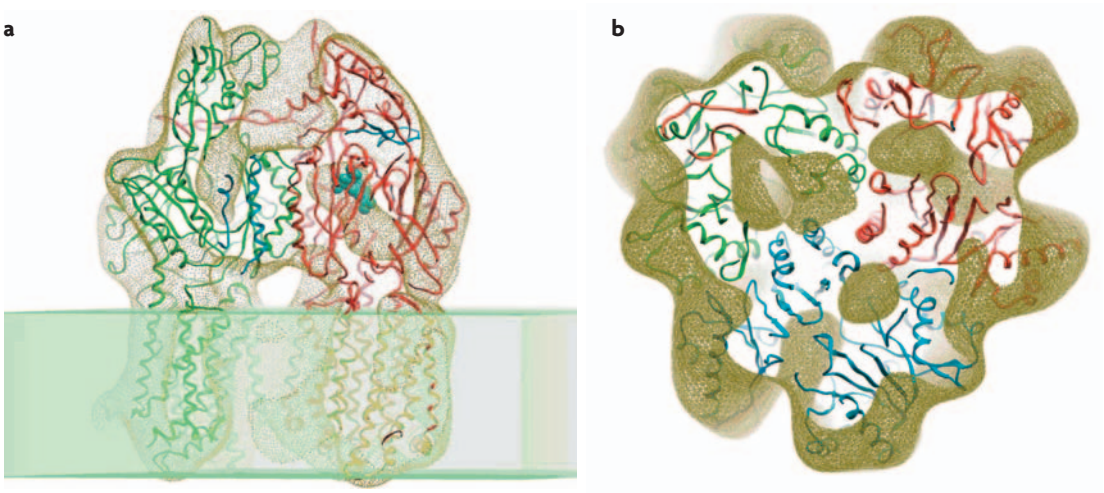

Figure 3 | **Structure of AcrB based on the asymmetric crystal. a** | Lateral view of the cross-section of the AcrB–substrate complex X-ray structure. Bound substrate (aquamarine space-filling model) can be observed in the cavity near the end of the deep gorge within the red subunit. The gorge has an opening to the periplasm on the right. The corresponding cavity in the green subunit is empty and opens to the crater-like structure at the top. Most of the blue subunit is removed by the cross-section. **b** | Top view of the transversal cross-section of the AcrB trimer X-ray structure. The three subunits are drawn as red, green and blue ribbons. The simplified shape of the protein surface (yellow mesh) was generated in ICM (Molsoft) using an FFT-based smoothing algorithm.

architecture of this binding pocket is very different from the one observed for AcrB. However, multidrug binding seems to obey the same rules.

In addition this structure provides important details on the mechanism of ATP-driven ABC-mediated efflux, which has been proposed to occur using an 'alternating access and release' mechanism. In essence, high-affinity substrate binding induces high-affinity ATP-binding, which in turn induces substrate release, ATP hydrolysis and subsequent release of ADP to re-set the system.

Returning to the subject of RND transporters, and as implied earlier, a substrate can reach the binding pocket through the uptake channel. The main entrance to the channel is about 15 Å above the plane of the membrane (FIG. 3a). It is easily accessible from the periplasmic space via the 'vestibules' leading to the central cavity. It seems possible that the role of the weak binding observed previously in the central cavity[68] might be to slow down the lateral diffusion of substrates in the vicinity of the entrance to the uptake channel.

Two recent studies, published simultaneously, provide further exciting and unexpected insights into possible transport mechanisms by RND transporters[39,40].

Waltzing pump: the alternate occupancy model

The first X-ray structure of the RND pump AcrB was presented as a perfectly symmetric trimer[35]. It gave rise to the so-called 'elevator mechanism'[68] of transport, which proposed that substrates accumulating in the central cavity are actively transported into the upper portal space via a channel that opens along the central axis of the structure. However, in this model a very significant conformational change associated with channel opening would have to be coupled with proton transport via the trans-membrane domain in order to accommodate the passage of substrates.

The two new structures of AcrB trimers, although symmetric overall, show each protomer in a distinct conformation[39,40]. Although only one of these 'new generation' structures contains co-crystallized substrates[39], the conformations of protomers in both are strikingly similar. This implies that substrate is not needed to induce asymmetry.

In the periplasmic portion, the main differences between the old and new models are in the substrate-binding pocket (FIG. 3b). First, the substrate is present in only one of the protomers, dubbed the 'binding' protomer (B). The spacious drug-binding pocket (described earlier) is open to the periplasm and expands far into the porter domain, almost reaching the TolC docking funnel. The exit from the pocket into the funnel is blocked by the inclined α-helix of the central pore from the adjacent protomer. The binding pocket of this second protomer, called the 'extrusion' protomer (E), is closed to the periplasm, significantly reduced in size and opened towards the funnel. The binding cavity of the third 'access' protomer (A) is largely inaccessible from either the periplasm or the exit funnel.

Based on this asymmetric structure, a new mechanism of drug transport has been proposed. This 'alternate occupancy' model implies that each protomer cycles through three consecutive conformations, named after F_1F_0-ATPase, as loose (L), tight (T) and open (O), corresponding to three phases of efflux[40]. This cycling is sequential, rather than synchronous, such that at any given time each protomer exists in a different phase.

Several sequences for a transport process can be envisioned. In one model[39,40], the first is the L-phase (corresponding to A-protomer), in which the substrate gains limited access to the uptake channel. During the second T-phase (corresponding to B-protomer), the uptake channel expands and the substrate enters the voluminous binding pocket. In the last O-phase (corresponding to

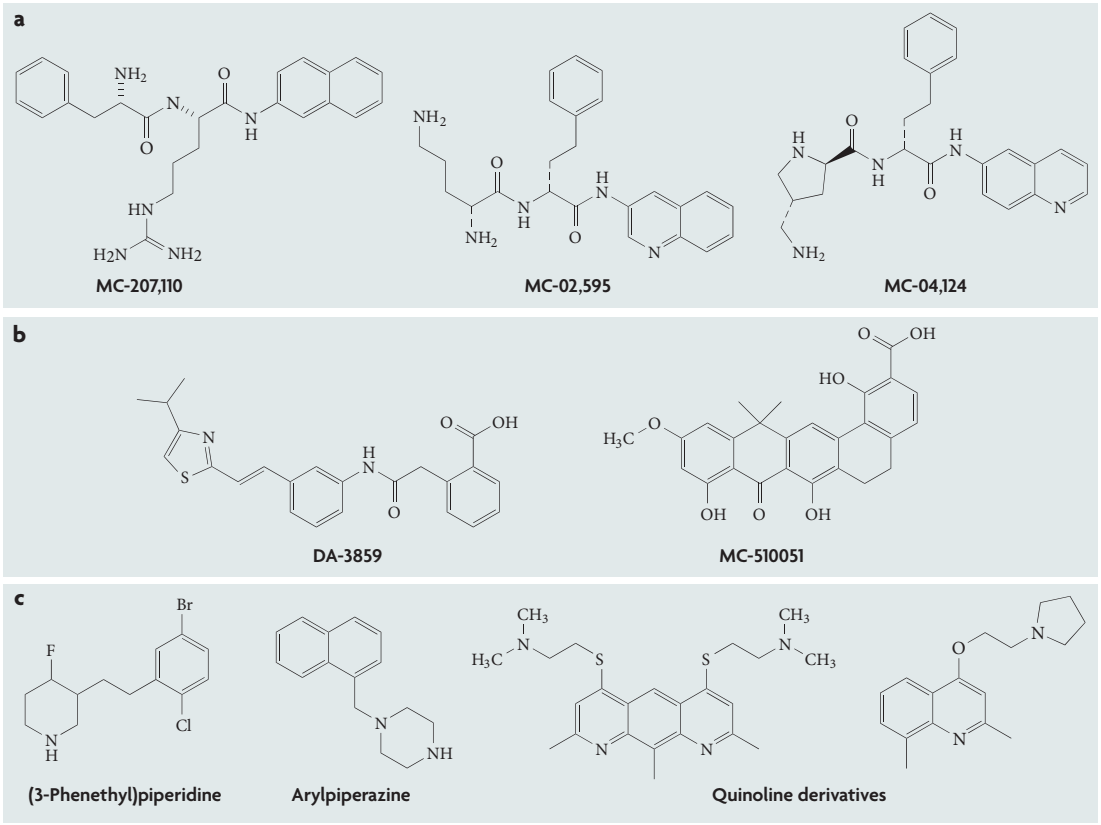

Figure 4 | **Various inhibitors of RND transporters. a** | Broad-spectrum efflux pump inhibitors (EPIs) with activity against multiple resistance-nodulation-cell-division (RND) pumps, including those in *Pseudomonas aeruginosa*. These inhibitors are themselves substrates of efflux pumps and most probably interact with the transporter via a substrate-binding pocket. **b** | Narrow-spectrum EPIs with selective activity against the MexAB–OprM complex from *P. aeruginosa*. These inhibitors might interact with the transporter at the allosteric 'modulator' site. **c** | EPIs with activity against transporters from various species of enterobacteria. Their mode of action is mostly uncharacterized.

the E-protomer), the binding pocket disconnects from the periplasm and shrinks in size. The drug is pushed out of the binding pocket (presumably concomitant with the α-helical exit opening) into the funnel, where it can diffuse into the TolC channel. At the same time one adjacent protomer receives another substrate molecule in the binding pocket, while the third protomer returns to the substrate-accepting state.

It should be noted that the role of the L-phase, in which the cavity is largely disconnected from either the periplasm or the funnel (FIG. 3b), is somewhat unclear. Rather than being the first, substrate-accepting phase, it might actually be the second. Transport might be initiated by binding into the expanded pocket after entering through the open uptake channel (as in the 'red' subunit on FIG. 3b). After a conformational change, it is possible that the cavity becomes closed off from the periplasm, entering the L-phase. It might be proposed that the substrate is then trapped but unbound in the cavity before the transition to the funnel-open state, when it is released. The volume of the closed cavity is estimated at ~1,200 Å^3, sufficient to accommodate substrates over a molecular mass of 1,000 Da. Finally, another possibility is that this is in fact the third and truly ligand-free phase following drug binding and drug release.

Based on the obvious analogy, AcrB has been nicknamed a peristaltic pump[40]. Perhaps a more poetic comparison would be with the three-step rotation of the Vienna Waltz. During the 'dance' each protomer of AcrB functionally rotates through three different positions. This model of RND-mediated efflux is also reminiscent of an exit through 'revolving doors'.

Regardless of the sequence of events that produce efflux of the substrate against the concentration gradient, the affinity of the substrate to the periplasm-accessible conformation of the pump subunit cavity is expected to be higher than the affinity to the funnel-opened conformation. The transition of the ligand-bound protomer from a high-affinity to low-affinity state should require energy input, which is evidently provided by the coupled proton transport in the trans-membrane domains. Although the details of the mechanism remain to be clarified, existing studies provide some initial clues.

Mutagenesis data indicate that AcrB has four electrostatically interactive residues that constitute the putative proton relay pathway, located in TM4, TM10 and TM11[82,83]. In the binding and access protomers, Lys940 of TM10 and Asp407 and Asp408 of TM4 are coordinated by salt bridges. However, in the extrusion protomer, Lys940 is turned toward Thr978 and the salt

bridges are absent. This in turn causes twisting of TM4 and TM10. Without additional data, it is impossible to say whether or not these subtle changes in TMDs are sufficient to produce the large conformational changes in the porter domain, which ultimately results in efflux. However, what is clear is that the residues involved in proton and substrate translocation are far apart.

Understanding the mechanism of transport and identification of the multidrug-binding pockets will help to design new clinically relevant approaches to inhibit drug efflux.

Targeting efflux pumps

It is clear from the discussion above that the inhibition of efflux would have several beneficial consequences. Efflux-pump inhibitors (EPIs), when administered in combination with antibiotics, enhance antibiotic potency. In addition, EPIs can expand the spectrum of an antibiotic to include previously non-susceptible species. These multifactorial benefits should override the difficulties of development of combination therapy. And such difficulties do exist; the development of any drug based on a new chemical entity (NCE) is a daunting task. In this regard, an efflux pump inhibitor is no different from any other NCE. However, the development of combination therapy brings additional complexity owing to the necessity for precision tailoring of the pharmacokinetics of both agents in order to achieve the desired pharmacodynamic effect.

It is also desirable that the antibiotic and the EPI should not engage in drug–drug interactions. In this respect some lessons can be learned from the clinical experience with inhibitors of P-gp and other ABC transporters as reversing agents for combination with anticancer drugs. The search for such compounds started in the mid-1970s, almost concomitant with the discovery of P-gp[1,84–88]. Several P-gp inhibitors have since failed in clinical trials. Perhaps the main reason for this is that P-gp and some other ABC transporters have a distinct physiological function in the human body: they protect various cells from endogenous toxic metabolites and xenobiotics, as well as the cytotoxic anticancer drugs themselves. In addition, they participate in drug disposition. As a result, in the presence of P-gp inhibitors, exposure to the co-administered cytotoxic drug in normal cells increased, resulting in toxicity. Several more potent and selective agents are undergoing clinical development at the present time[76].

It is expected that the introduction of inhibitors of bacterial RND transporters as anti-infective agents to the clinic might be more expeditious than for MDR-reversing agents for cancer therapy, as no close human homologues of RND transporters exist, and therefore no target-based toxicity is expected.

In addition, based on the emerging significance of RND transporters in bacterial pathogenesis one can imagine EPIs as stand alone, antivirulence agents.

Different modes of transport inhibition

Several ways of interfering with the function of RND transporters are apparent following elucidation of the structure and transport mechanism.

Inhibitors can target the substrate-binding pocket. This voluminous pocket can accommodate an extraordinary variety of structures, and so many distinct pharmacophore classes can be foreseen. Importantly, the binding site of the RND transporter is located in the periplasm, so that an inhibitor might need to cross only one membrane.

As was evident in the recent structural study[39], various substrates can occupy different locations in the large substrate-binding pocket. It is therefore expected that specific inhibitors will interact with and potentiate only a subset of substrate antibiotics. This is the case with MC-207,110, the very first EPI to be identified, which is active against several RND pumps in a variety of Gram-negative pathogens. This compound (FIG. 4a) was originally identified in a screen for potentiators of the fluoroquinolone levofloxacin in the RND pump-expressing organism *P. aeruginosa*[21], and is now routinely used to evaluate the contribution of efflux in various Gram-negative bacteria[89]. Consistent with targeting the substrate-binding region of the pumps, the inhibitor potentiates the activity of only some antibiotic pump substrates (for example, fluoroquinolones, macrolides and tetracyclines, but not β-lactams) and was itself shown to be exported by RND systems[21]. More advanced analogues of this compound were also identified in a collaboration between Microcide Pharmaceuticals and Daiichi Pharmaceuticals (for example, MC-02,595,2[90] and MC-04,124[91], FIG. 4a), and Mpex Pharmaceuticals is pursuing an EPI programme based on inhibitors from this class of compounds. Recently, a series of aryl-piperazines (FIG. 4c) were identified as inhibitors of at least two RND systems from *E. coli* (AcrAB-TolC and AcrEF-TolC[92]) and other RND pumps in enterobacteria[93]. As with MC-207,110, they seem to potentiate the activity of some, but not all, of the antimicrobial substrates of these transporters. Interestingly, they differ from MC-207,110 in the spectrum of the antibiotics affected[93].

Importantly, an EPI interacting with the RND transporter in the binding site can have different modes of inhibition. It can compete directly for binding with other substrates, but it can also facilitate such binding by virtue of positive cooperativity and thereby prevent dissociation of substrates from the pump.

In addition, based on the structure of AcrB, two of the three different conformations of the binding pocket offer the potential for inhibition without requiring that the drug reach the cytoplasm. The T-phase (or B-protomer) conformer might be accessed from the periplasm, and the O-phase (or E-protomer) from the TolC-docking funnel. These two conformations might be targeted independently.

Efflux inhibitors might also act at sites distinct from those involved in substrate binding but whose disruption impacts overall pump activity. Such allosteric inhibitors would be expected to inhibit efflux of all substrates and therefore potentiate the activity of multiple antibiotics. A series of structurally diverse inhibitors with high selectivity towards the MexAB–OprM efflux pump from *P. aeruginosa* have been identified[94–99] (FIG. 4b) and shown

to negatively affect the export of all MexAB–OprM antimicrobial substrates equally[94]. It was proposed that these EPIs bind not to substrate-binding sites on the pump, but rather to site(s) that modulate pump activity (that is, modulation sites). Several alkoxy- and alkylaminoquinoline EPIs (FIG. 4c) showing activity against clinical strains of *Enterobacter aerogenes* have been reported[100–103]. These potentiate the activities of all antimicrobials tested equally, consistent with action at a modulation site of an RND-type efflux system.

At present, it is unclear whether or not the RND transporters do, in fact, have a 'dedicated' modulation site, but the empirical observation of a link between the capacity to potentiate multiple substrates ('modulator mode') and high selectivity towards specific RND transporters is suggestive of such a feature.

Other possibilities for interfering with efflux include targeting the assembly of the pump components and blocking the TolC-like tunnel, although these are purely hypothetical as there are no reports of molecules with such activity yet.

The search for efflux inhibitors

The structural elucidation of efflux pumps will undoubtedly facilitate the future search and optimization of EPIs. It will also help to clarify the mode of action of EPIs identified in the past using more traditional approaches.

Up to now, most inhibitors of efflux pumps have been discovered through traditional random screening of synthetic compounds or natural-product libraries. The assays that are used are very simple and can easily be adapted to high-throughput formats[21]. For example, MC-207,110 was identified in a levofloxacin potentiation assay using a strain of *P. aeruginosa* overexpressing MexAB–OprM, whereas (3-phenethyl)piperidines[104] (FIG. 4c) were found by scientists at Pharmacia (now Pfizer) in a novobiocin potentiation assay using an AcrAB–TolC-overexpressing strain of *E. coli*.

An alternative approach is to screen libraries of known drugs. The identification of a novel mode of action in an approved drug could significantly shorten the development pathway and mitigate the risks inherent in an NCE. However, for such a proposition to be practical, the EPI activity needs to be much higher in potency compared with the original pharmacological activity. An interesting possibility would be to discover an EPI mode of action against RND transporters in compounds that themselves are anti-infective agents but are used for a different indication. One such compound, MP-601,205[23], was identified by scientists at Mpex. This compound entered Phase I clinical trials in cystic fibrosis patients, but the programme is currently suspended because of concerns about drug tolerability.

Recently, several efficient pharmaco-informatic methods have been reported for the identification of new inhibitors of P-gp[85]. Leads were discovered by ligand-based virtual screening of large, commercially available libraries of compounds. These approaches are based on machine-learning algorithms and require a relatively large number of known inhibitors to be used as a training set. In the case of P-gp, many structurally unrelated compounds are available for this purpose. It is expected that as an increasing number of more diverse bacterial EPIs are identified through 'wet' screening, the more feasible alternative screening methods, including various virtual screening protocols, will become.

Finally, based on the characteristics of the binding sites of RND pumps, another approach might be the synthesis of flexible molecules carrying multiple aromatic moieties that could bind with high affinity into recognition cavities by inducing the best fit in the binding pocket. A complementary approach might be the synthesis of inhibitor dimers[105] and substrate–inhibitor hybrids.

Continuing efforts in both academic and applied research should help introduce these important agents into clinical practice. Demonstration of their multifactorial benefits in clinical settings will provide the ultimate validation of the EPI-based combination approach.

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Competing interests statement
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